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The Biosynthesis of 4-Hydroxycoumarin and Dicoumarol by *Aspergillus fumigatus* Fresenius

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A strain of *Aspergillus fumigatus* Fresenius, isolated from spoiled hay, converts melilotic acid (*o*-hydroxyphenylpropionic acid) and *o*-coumaric acid into 4-hydroxycoumarin and dicoumarol. The sequence is shown to be melilotic acid (I) $\xrightarrow{-2H}$ *o*-coumaric acid (IV) $\xrightarrow{+H_2O}$ β-hydroxymelilotic acid (II) $\xrightarrow{-2H}$ β-oxomelilotic acid (III) $\longrightarrow$ 4-hydroxycoumarin (VI), on the basis of (1) studies on the formation of postulated intermediates, (2) experiments with isotopically labelled materials and (3) sequential enzyme induction. In the presence of semicarbazide, *o*-coumaraldehyde is formed from *o*-coumaric acid: there is no evidence, however, that this lies on the normal metabolic pathway.

Sweet clover (*Melilotus alba*) or sweet vernal (*Anthoxanthum odoratum*) occasionally undergo spoilage during storage, associated with moulding and 'fermentation' of the resulting hay. The temperature may rise to 60°C with production of the haemorrhagic agent dicoumarol (VII) (Stahman, Heubner & Link, 1941). Melilotic acid (*o*-hydroxyphenylpropionic acid) (I) and *o*-coumaric acid (IV) both present in *A. odoratum*, can serve as precursors for dicoumarol production (Bye, Ashton & King, 1968). 4-Hydroxycoumarin (VI) can react non-enzymically with formaldehyde to form dicoumarol (Spring & Stoker, 1968). Although the conversion of *o*-coumaric acid into 4-hydroxycoumarin has been observed several times (Bocks, 1967; Bellis, Spring & Stoker, 1967; Shieh & Blackwood, 1967) little work has been carried out on the mechanism. It seemed possible that a β-oxidation mechanism was involved (Bye *et al.* 1968; Spring & Stoker, 1969) and this paper describes a search for intermediates.

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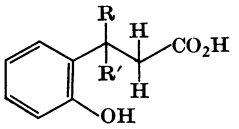
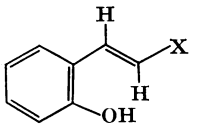
MATERIALS AND METHODS

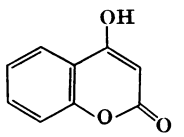
Reagents. Analytical-grade solvents and reagents were used whenever available, otherwise reagent-grade chemicals recrystallized to constant melting point were used. Diethyl ether was stored over sodium and redistilled



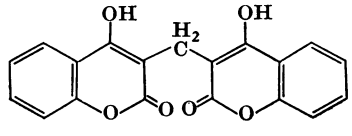
(I; R = H, R′ = H) Melilotic acid
(II; R = OH, R′ = H) β-Hydroxymelilotic acid
(III; R = R′ = oxo) β-Oxomelilotic acid



(IV; X = CO_2H) *o*-Coumaric acid
(V; X = CHO) *o*-Coumaraldehyde



(VI) 4-Hydroxycoumarin



(VII) Dicoumarol

over reduced iron immediately before use. Deionized water was used throughout unless otherwise stated.

β-Hydroxymelilotic acid and β-oxomelilotic acid. We acknowledge generous gifts of these compounds from Dr M. S. Spring (Spring & Stoker, 1969).

[2-^{14}C]o-*Coumaric acid.* A 2.5μl portion of salicylaldehyde (purified by preparation of the copper acetate complex; Vogel, 1955) was heated under reflux for 6h with 100μCi of [2-^{14}C]malonic acid (The Radiochemical Centre, Amersham, Bucks., U.K.) diluted to 10mg with unlabelled malonic acid, in 100μl of pyridine with addition of 5μl of piperidine as catalyst (Dutt, 1924).

On cooling, the reaction mixture was acidified with 0.25ml of 6M-HCl and applied as a band to a thin-layer chromatogram (see below) which was developed with solvent system 1. The band associated with *o*-coumaric acid was eluted with ethanol and this procedure was repeated with solvent systems (3*a*) and (3*b*). After elution, the resulting *o*-coumaric acid had a specific radioactivity of 14.1μCi/mg (yield 1.9mg; 31% based on salicylaldehyde) ϵ_{275}^{EtOH} 16500 (ϵ_{275}^{EtOH} 16500 for authentic *o*-coumaric acid).

[G-^{3}H]o-*Coumaric acid.* A 25mCi (3.3mg) portion of [G-^{3}H]coumarin (The Radiochemical Centre) was added to 246.7mg of unlabelled coumarin. The mixture was quantitatively converted into *o*-coumaric acid by the method of Seshadri & Roa (1954) (the ^{3}H label is very stable when associated with a benzene ring; Evans, 1966). The specific radioactivity of the resulting *o*-coumaric acid was 0.103mCi/mg (yield 278mg); ϵ_{275}^{EtOH} 16600.

Isolation and culture of micro-organisms. The basal medium contained: $NaNO_3$ (6g), KCl (0.52g), $MgSO_4,7H_2O$ (0.59g), KH_2PO_4 (1.52g), $FeSO_4,7H_2O$ (4mg), $ZnSO_4,7H_2O$ (0.4mg), glucose (10g) and water to 1 litre, pH4.8 (after sterilization).

For isolation of organisms from hay and maintenance of stock cultures, basal medium was solidified with 1.5% (w/v) of Ionagar no. 2 (Oxoid Ltd., London E.C.4, U.K.). Rose Bengal (15p.p.m.) and streptomycin (15p.p.m.) were added as selective bacterial inhibitors in all agars used for pure fungal cultures. Agar and liquid medium were sterilized by autoclaving at 120°C for 20min.

A 5g portion of moulding hay (*A. odoratum*) was shaken with 25ml of sterile water, and 1.0ml of supernatant fluid was transferred aseptically to basal medium plus 0.005% *o*-coumaric acid (100ml in a 250ml conical flask). The flask was incubated on a rotary shaker at 55°C (the normal temperature of moulding hay actively producing dicoumarol).

When dicoumarol was found to be present in the medium (after 72h) loopfuls of the culture were plated out on to basal agar plus 0.005% *o*-coumaric acid and after incubation (at 55°C for 72h) colonies were identified as (*a*) bacteria, (*b*) fungi or (*c*) actinomycetes. Single colonies from each group were inoculated into 50ml of basal medium plus 0.005% *o*-coumaric acid and incubated (at 55°C for 72h) on a rotary shaker; the medium was then examined for dicoumarol and 4-hydroxycoumarin as described below.

Of five bacteria isolated only two (identified as pseudomonads) metabolized *o*-coumaric acid, producing melilotic acid, not dicoumarol, which supports the findings of Levy & Weinstein (1964). Two organisms from the actinomycete group gave both poor growth and no metabolism of *o*-coumaric acid. Four organisms from the fungal group all metabolized *o*-coumaric acid to dicoumarol. However, one organism (later identified by the Commonwealth Mycological Institute, Kew, U.K., as *Aspergillus fumigatus* Fresenius) gave far better yields than the rest and was chosen for subsequent studies.

Utilization of substrates. The *A. fumigatus* master agar slope was stored at 2°C and subcultured monthly (Roberg, 1948). Sub-master slopes inoculated from the master slope were incubated at 45°C for 72h. The sub-master slope was then washed with 5ml of sterile basal medium, which was used to inoculate the liquid medium (about 10^5 conidia/100ml of medium). The inoculated medium was normally incubated for 36–72h at 45°C on a rotary shaker (the temperature of 45±2°C was taken as a convenient working temperature as 55°C was difficult to maintain with the equipment used). All incubations were carried out in the dark as strong illumination caused *cis–trans* isomerization. The metabolism of the following was studied: (*a*) *o*-coumaric acid; (*b*) melilotic acid; (*c*) β-hydroxymelilotic acid; (*d*) β-oxomelilotic acid; (*e*) 4-hydroxycoumarin; (*f*) dicoumarol. Two methods were used. (1) The substrate (5mM) was added to the basal medium at the time of conidial inoculation. Utilization of the substrate commenced only after 36–48h, during which time the pH rose from 4.8 to 7.0. (2) Basal medium was inoculated with conidia and incubated at 45°C for 48h. The mycelium was filtered free of spent medium, washed once with fresh medium without glucose (adjusted to pH6.5–7.0) and then resuspended in medium (pH6.5–7.0) containing the substrate (5mM) but no glucose. Utilization of the substrate began after 1.5–4hr. Metabolism was measured by removal of the starting material and by formation of products. When radioactively labelled materials were used the conversion of as little as 10^{-5} of the added substrate could be detected.

Separation and examination of metabolites (*Table* 1). A 1.0ml portion of culture (containing about 800μg of substrate and metabolites) was acidified to pH2 with 6M-HCl and both organisms and medium were extracted together with 5vol. of ether. The ether was separated, dried over anhydrous Na_2SO_4 (2–3g) for 2–4h and evaporated to dryness. The residue, taken up in a minimal amount of ether, was applied across the origin of a t.l.c. plate (20cm×20cm, thickness 0.25mm) of Kieselgel G (E. Merck A.-G., Darmstadt, Germany), which was irrigated (ascending) with one of the following solvent systems (Stahl, 1965) until the solvent front had travelled about 10cm; (1) benzene–dioxan–acetic acid (90:25:4, by vol.); (2) propan-1-ol–aq. NH_3 (sp.gr. 0.88)–water (7:2:1, by vol.); (3) two-dimensional system, (*a*) chloroform–ethyl acetate (4:1, v/v), (*b*) benzene–ethyl acetate–formic acid (18:1:1, by vol.).

The chromatograms were dried in a stream of warm air and then examined under u.v. light at 260nm and 360nm (Hanovia Chromatolight). The sample chromatogram was then divided into three portions, which were sprayed respectively with: (i) Rhodamine 6G [BDH (chemicals) Ltd., Poole, Dorset, U.K.] (0.01%, w/v) in acetone; (ii) Gibb's reagent (2,6-dibromo-*p*-benzoquinone-4-chlorimine) as a 0.1% (w/v) solution in methanol, followed by an overspray of saturated $NaHCO_3$ solution; (iii) Brentamine Fast Blue B salt (BDH) (0.1%) in water. Duplicate chromatograms of authentic substances were similarly

Table 1. *Identification of compounds*

Details of solvents and spray reagents are given in the Materials and Methods section.

	R_F values				Colour reactions: U.v. characteristics			Colour reactions		Spectrophotometric data	
	Solvent 1	Solvent 2	Solvent 3*a*	Solvent 3*b*	260nm	360nm	Rhodamine 6G, 260nm	Gibb's reagent	Brentamine Fast Blue B salt	$\lambda_{max.}^{EtOH}$ (nm)	ϵ
Melilotic acid	0.50	0.65	0.27	0.40	—	Quenching	Quenching	Dark blue	Pink	275	2100
β-Hydroxymelilotic acid	0.28	0.75	0.38	0.25	—	Quenching	Quenching	Dark blue	Orange	274	2512†
										278 (infl.)	1413†
β-Oxomelilotic acid	0.40	0.60	0.32	0.50	Faint yellow	Yellow	Faint quenching	Dark blue	Yellow	256	9772
										331	3548
o-Coumaric acid	0.46	0.45	0.25	0.42	Silver	Blue	Faint quenching	Light blue	Yellow-brown	274	16600
										320	8400
o-Coumaraldehyde	0.44	0.40	0.21	0.45	Blue	Blue	Faint quenching	Light blue	Yellow-brown	270	16200
										320	6320
Coumarin	0.80	0.94	0.78	0.58	—	Quenching	Quenching	Yellow brown	—	275	12300
										300	6150
4-Hydroxycoumarin	0.35	0.70	0.17	0.10	Quenching	Quenching	Quenching	Purple	Violet-red	280	10200
										288	9900
										305	10000
Dicoumarol	0.72	0.80	0.58	0.64	—	Quenching	Quenching	—	Dark red	289*	21200
										310*	23460
										325*	19320

* Spectrophotometric data for dicoumarol obtained in chloroform.
† Determined by Dr M. S. Spring.

treated. Examination under the u.v. lamp or treatment with the various sprays were equally sensitive, allowing detection down to about 5 μg.

For further examination plates were run without dye treatment except for a strip at the edge for location of the required band. The Kieselgel (about 250 mg) was scraped off and suspended in 5 ml of water, then extracted into ether (3 × 10 ml); recovery of known amounts of authentic compounds was over 80%. Compounds were measured by their u.v. absorption in ethanolic solution, by using molar extinction values determined on authentic compounds unless otherwise indicated. Compounds were identified by comparison of R_F values with authentic standards, and by colour reactions, u.v.- and i.r.-absorption spectra and mass spectra.

Glucose and reducing sugars. These were measured by the anthrone reaction (Fairbairn, 1953).

Soluble protein. This was measured by the biuret reaction (Gornall, Bardawill & David, 1949).

Isolation and identification of keto compounds. The fungus was grown at 45°C for 36 h in 1 litre of basal medium with *o*-coumaric acid (5 mM) and harvested by aseptic centrifugation (10000*g* for 10 min). The combined pellets (200 g wet wt.) were resuspended aseptically in 500 ml of sterile basal medium containing semicarbazide (10 mM) and *o*-coumaric acid (5 mM) and adjusted to pH 6.5 with 2 M-NaOH, in a 2 l conical flask. The suspension was incubated at 45°C for 48 h on a rotary shaker. The mycelium and medium were treated with the Ultra-Turrax gun homogenizer (Janke und Kunkel K.G., Staufen i. Br., Germany) for three 5 min periods and the resulting fungal debris was filtered off through four layers of cheesecloth, and 250 ml of saturated 2,4-dinitrophenylhydrazine in 2 M-HCl was added to the filtrate. The resulting precipitate (0.8 g) was collected by filtration and dried in a stream of warm air. The dried residue (0.5 g) was dissolved in a minimal quantity of ethyl acetate (about 2.0 ml) and applied to a 50 g column (50 cm × 1.5 cm) of neutral (Brockmann grade III) type H alumina (Gallenkamp Ltd., Widnes, Lancs., U.K.). The column was developed with 100 ml of ethyl acetate followed successively with 1%, 3%, 5% and 10% (v/v) ethanol in ethyl acetate. Each eluate fraction was evaporated by distillation under atmospheric pressure, and the free carbonyl compounds were displaced from the 2,4-dinitrophenylhydrazones by addition of benzaldehyde (Schmidt & Treiber, 1933) and examined spectrophotometrically in ethanolic solution. Only the material eluted with 5% ethanol in ethyl acetate showed the 275 nm and 306–325 nm extinctions characteristic of coumarins: it was purified by t.l.c. by using successively the three solvent systems described in Table 1. The yield was 0.1 g.

Formation of xanthates. A cell-free extract was prepared by inoculating 2 × 500 ml of basal medium plus 0.005% (w/v) *o*-coumaric acid, in 2 l flasks, with conidia and incubating at 45°C for 48 h in shake culture. The mycelium was harvested by suction filtration through two layers of cheesecloth, resuspended in 0.05 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7.0, and refiltered. The fungal mat (50 g wet wt.), still moist with buffer, was crushed under liquid N_2 to a fine powder. The powder from the fungus was left to thaw for 4–6 h at 2°C and coarse debris was removed by centrifugation at 1000*g* for 20 min. The cloudy supernatant, containing between 6 and 20 mg of protein/ml and also the enzyme system, could oxidize *o*-coumaric acid to 4-hydroxycoumarin.

Hydroxamate production was estimated by the $FeCl_3$ reaction (Stadtman, 1955*b*). Standard *o*-coumaric acid and melilotic acid hydroxamates were prepared by the method of Thompson (1951). The incubation procedure was that of Stadtman (1955*a*); crude enzyme preparation containing 20 mg of protein in 10 ml of 250 μM-sodium–potassium phosphate buffer, pH 7.0, with 200 μM-$MgCl_2$, 200 μM-ATP, 2 mM-neutral hydroxylamine and 100 μM substrate were incubated at 37°C for 0–36 h.

For identification of hydroxamates 1.0 ml of the incubation mixture was discharged into 25 ml of ethanol. The protein precipitate was removed by centrifugation (2000*g* for 10 min) and the supernatant evaporated to dryness by vacuum distillation. The residue (10–30 mg) was taken up in 0.2 ml of methanol and spotted on to a t.l.c. plate (0.25 mm thick) of Kieselgel G. The plate was developed with solvent 1 and spots were detected with a spray of 5% (w/v) $FeCl_3$ in ethanol. Spots were eluted by extracting 50 mg of the gel with 10 ml of ethanol.

Sequential enzyme induction. The substantial lag observed in cultures before detectable metabolism of *o*-coumaric acid or melilotic acid commenced suggested that an inducible enzyme system might be involved. If so, some indication of the sequence may be obtained by applying the concept of 'sequential induction' (Stanier, 1951), through determining whether the lag could be abolished or decreased by previous exposure to either of the two 4-hydroxycoumarin precursors.

The fungus was grown in 200 ml of basal medium plus melilotic acid (5 mM) for 48 h at 45°C, then the mycelium was filtered off, washed aseptically with basal medium and resuspended in fresh sterile mineral salts medium, pH 7 (100 ml), containing 5 mM-*o*-coumaric acid or 5 mM-melilotic acid but no other carbon source. In a similar experiment fungus was grown in basal medium plus *o*-coumaric acid (5 mM) before washing and resuspension into fresh mineral salts medium, pH 7, containing 5 mM-*o*-coumaric acid or 5 mM-melilotic acid.

Radioactive tracer experiments. *o*-Coumaric acid labelled with 5 μCi of ^{14}C or 250 μCi of ^{3}H was added/100 ml of culture. Fractions were isolated and identified as described above. ^{14}C and ^{3}H were measured in a Packard Tri-Carb model 314 EX liquid-scintillation counter. The scintillator used was toluene with 6 g of 2,5-diphenyloxazole/l as primary scintillator and 0.05 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l as secondary scintillator. No correction was needed for quenching and the efficiency of the counter was about 50% for ^{14}C and 14% for ^{3}H. The efficiency of the machine was checked for each experiment by using a known ^{14}C or ^{3}H standard.

Thin-layer chromatograms were scanned for radioactive compounds by using a Desaga scanner 12-2 (Rank, Murphy, Bush Instruments, London W.4, U.K.).

Fractions that showed radioactivity but contained insufficient material for identification by normal methods were characterized as follows. A series of incubation mixtures was set up in the usual way, and after separation of the organism authentic samples of compounds that were likely to be identical with the unknown material were added. The added material was isolated by t.l.c. as described above. If the radioactivity was associated with the recovered compound, the fraction was rechromato-

Table 2. *Effect of* o-*coumaric acid and melilotic acid concentration on growth and metabolism of the organism*

	Inoculated with conidia		Treatment with pregrown fungus	
Concn. of *o*-coumaric acid or melilotic acid (mM)	Concn. of 4-hydroxycoumarin after 48 h (mM)	Conidia germination	Concn. of 4-hydroxycoumarin after 48 h (mM)	Ability to inoculate fresh medium
0.1	0.1	+	0.1	+
1.0	0.8	+	1.0	+
10	0.0	−	1.0	+
100	0.0	−	0.0	−

Table 3. *Utilization of* o-*coumaric acid and of melilotic acid by* A. fumigatus

Basal medium plus 5 mM-*o*-coumaric acid or 5 mM-melilotic acid was inoculated with conidia and incubated at 45°C on a rotary shaker.

Time after conidial inoculation (h)	Concn. of glucose (mM)	Concn. of *o*-coumaric acid or melilotic acid (mM)	Concn. of 4-hydroxycoumarin (mM)	Wet wt. of fungus (g)	pH
o-Coumaric acid					
0	555	5.0	0.0	0	4.8
4	402	4.2	0.0	0.10	5.1
16	135	2.7	0.0	0.30	5.6
24	10	1.5	0.3	0.45	6.2
36	5	1.0	2.5	0.52	6.8
48	3	0.0	5.0	0.55	6.8
72	1	0.0	4.8	0.60	7.0
Melilotic acid					
0	555	5.0	0.0	0	4.8
4	505	5.0	0.0	0.05	4.8
16	350	4.4	0.0	0.25	5.3
24	102	3.0	0.1	0.40	6.0
36	8	1.2	1.8	0.46	6.5
48	4	0.4	3.4	0.55	6.8
72	1	0.0	4.9	0.58	7.0

graphed with solvent systems 1, 2 and 3 until a constant specific radioactivity was obtained.

RESULTS

Conversion of o-*coumaric acid and melilotic acid into 4-hydroxycoumarin* (*Table* 2). *o*-Coumaric acid at 1 mM was converted almost quantitatively into 4-hydroxycoumarin in *A. fumigatus* under the growth conditions specified. At higher concentrations growth was inhibited. Under 'washed suspension' conditions conversion was observed up to 10 mM-*o*-coumaric acid, though the rate of formation of 4-hydroxycoumarin was no greater than at 1 mM. Higher concentrations of *o*-coumaric acid were inhibitory or lethal.

Table 3 shows that growth of the mycelium was accompanied by a rise in pH, but metabolism of *o*-coumaric acid did not commence until several hours after the start of glucose metabolism. Between 4 and 48 h the disappearance of *o*-coumaric acid cannot be accounted for by 4-hydroxycoumarin production, which strongly suggests that some intermediate(s) are being formed over this time. The pattern of utilization of melilotic acid was very similar.

Sequential enzyme induction (*Fig.* 1). Mycelium grown in basal medium metabolizes melilotic acid and *o*-coumaric acid only after a lag of 36–48 h. When melilotic acid is present in the growth medium, both substrates are metabolized without significant lag. When *o*-coumaric acid was present during growth it is subsequently metabolized without lag, but melilotic acid metabolism commences only after a lag of 4–6 h. The sequence therefore appears to be:

Melilotic acid → *o*-coumaric acid → 4-hydroxycoumarin

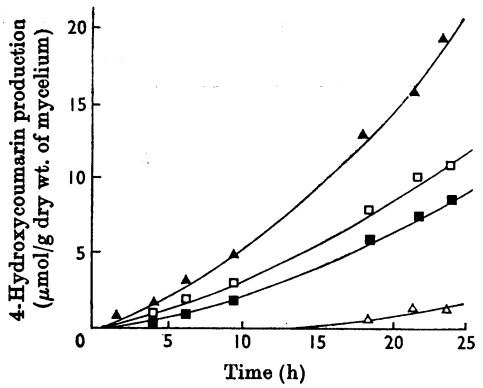


Fig. 1. Production of 4-hydroxycoumarin by *A. fumigatus*. Melilotic acid or *o*-coumaric acid was present at a concentration of 5 mM in both growth medium and metabolic solution. There was no detectable 4-hydroxycoumarin production when mycelium grown on *o*-coumaric acid or melilotic acid was transferred to a metabolic solution containing neither *o*-coumaric acid nor melilotic acid. Cells were grown on *o*-coumaric acid medium and then transferred to *o*-coumaric acid (▲) or melilotic acid (△) medium. Also cells were grown on melilotic acid medium then transferred to melilotic acid (■) or *o*-coumaric acid (□) medium.

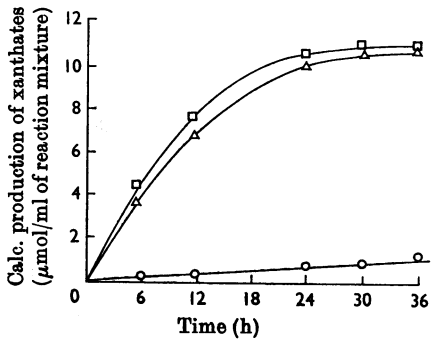


Fig. 2. Esterification of *o*-coumaric acid and melilotic acid by a fungal extract. Samples (1.0 ml) were withdrawn from a 10 ml reaction mixture containing 100 μM-*o*-coumaric acid (□), 100 μM-melilotic acid (△) and a no-substrate control (○), and then xanthates measured by the $FeCl_3$ colour reaction, by using standard curves. An E_{340}^{EtOH} value of 1.0 was equivalent to 16.4 g of methyl *o*-coumarate or methyl melilotate.

Esterification of o-*coumaric acid and melilotic acid.* *o*-Coumaric acid and melilotic acid are quantitatively esterified by fungal extracts as shown by the formation of the corresponding xanthates (Fig. 2). These were shown by t.l.c. to be the only xanthates present in substantial amounts (*o*-coumaryl hydroxamate, R_F 0.39; melilotyl hydroxamate, R_F 0.47); the other faint bands observed on the chromatograms were attributable to the coloured derivatives of ferric chloride with the corresponding lactones (coumarin, R_F 0.76; 3,4-dihydrocoumarin, R_F 0.81) and also endogenous metabolism (R_F 0.1, with tailing).

Semicarbazide derivatives of intermediates. When mycelium was incubated with *o*-coumaric acid, in the presence of semicarbazide, the only carbonyl derivative obtained (apart from those attributable to common metabolic intermediates, e.g. pyruvic acid) was isolated as *o*-coumaraldehyde 2,4-dinitrophenylhydrazone. This was not obtained from corresponding incubations with melilotic acid. *o*-Coumaraldehyde recovered from the 2,4-dinitrophenylhydrazone (see the Materials and Methods section) was identified by comparison with authentic material (Table 4). (1) The u.v.- and i.r.-absorption spectra were identical with those of *o*-coumaraldehyde prepared synthetically by the method of Pauly & Wascher (1923). (2) In the mass spectrogram the heaviest ion had a mass number of 148, and the cracking pattern followed closely that of the authentic compound.

Metabolism of labelled o-*coumaric acid.* [2-^{14}C]- or [G-^{3}H]-*o*-coumaric acid were added to basal medium, which was then inoculated with the organism and incubated. Samples were withdrawn and unchanged *o*-coumaric acid, β-hydroxymelilotic acid, β-oxomelilotic acid and 4-hydroxycoumarin were separated and their radioactivity determined as described above (Table 5).

Conversion of *o*-coumaric acid into 4-hydroxycoumarin occurs almost quantitatively and it seems that any further transformation of the latter compound must be very slow under the conditions of our experiments.

The complete recovery of ^{14}C shows that the side chain is not involved in any significant breakdown or exchange with the organism's metabolic pool. In interpreting the results for ^{3}H it must be remembered that the radioisotope is largely confined to the benzene ring, so the loss of hydrogen from the side chain is not reflected in these values, which confirm, as would be expected, that no metabolism of the benzene ring occurs.

DISCUSSION

The results presented, taken in conjunction with those of previous authors, suggest that the formation of dicoumarol from melilotic acid and *o*-coumaric acid takes place through the reaction sequence outlined in Scheme 1. The last stage, formation of dicoumarol from 4-hydroxycoumarin, is believed to be a slow non-enzymic reaction (Spring & Stoker,

Table 4. *Comparison between authentic* o-*coumaraldehyde and the isolated* o-*coumaraldehyde*

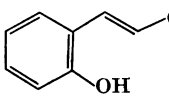
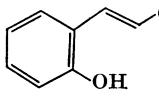
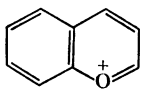
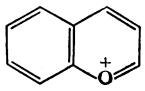
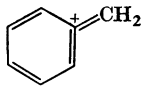
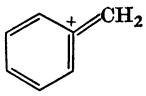
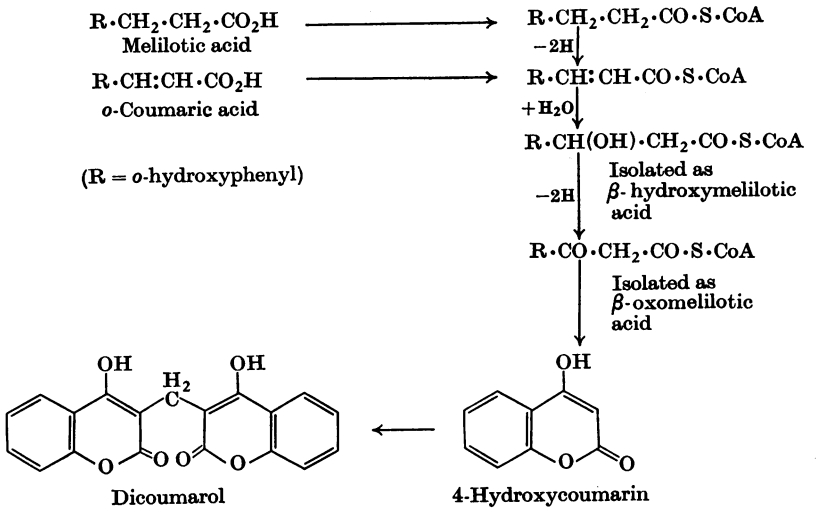
	Authentic *o*-coumaraldehyde		Isolated *o*-coumaraldehyde	
(i) U.v. characteristics				
	$\lambda_{max.}^{EtOH}$ (nm)	ϵ	$\lambda_{max.}^{EtOH}$ (nm)	ϵ
	270	14800	270	14700
	275	15600	275	15600
	325	8200	324	8080
(ii) I.r. characteristics				
	Characteristic groups	Wavenumber (cm^{-1}) of absorption	Characteristic groups	Wavenumber (cm^{-1}) of absorption
	–OH (free)	3600	–OH (free)	3600
	C–H (aldehyde)	2810, 2720	C–H (aldehyde)	2810, 2720
	C=O (aldehyde)	1700	C=O (aldehyde)	1690
	C=C, C=O	1600	C=C, C=O	1600
	C=C (*trans*)	995	C=C (*trans*)	995
	Aromatic ring with *o*-hydroxyl	750	Aromatic ring with *o*-hydroxyl	750
(iii) Mass spectrometry				
	Fragment	*m/e*	Fragment	*m/e*
		148		148
		131		131
		91		91
	$C_6H_5^+$	77	$C_6H_5^+$	77

Table 5. *Metabolism of* [G-^{3}H]o-*coumaric acid and* [2-^{14}C]o-*coumaric acid*

Medium (100 ml) containing 5 mM-[G-^{3}H]*o*-coumaric acid (250 μCi; sp. radioactivity 0.109 mCi/mg) or 5 mM-[2-^{14}C]*o*-coumaric acid (5 μCi; sp. radioactivity 14.1 mCi/mg) was inoculated with conidia and 5 ml samples were assayed after incubation for the times shown.

Time after inoculation (h)	Radioactivity (d.p.m.) *o*-Coumaric acid	β-Hydroxymelilotic acid	β-Oxomelilotic acid	4-Hydroxycoumarin	Wet wt. of cells (g)
[G-^{3}H]*o*-Coumaric acid					
0	2750000	0	0	0	0
18	1509000	1195000	520	0	0.18
36	1019000	698000	616000	107000	0.35
48	5200	130000	1750000	819000	0.48
72	630	990	150000	2500000	0.52
[2-^{14}C]*o*-Coumaric acid					
0	552380	0	0	0	0
18	155080	350000	418	38	0.3
36	108000	235350	103040	68880	0.55
48	116	259	211980	295600	0.59
72	12	35	402	545980	0.60



Scheme 1.

1968). The present authors have been concerned with the preceding steps.

The first step, the esterification of melilotic acid and *o*-coumaric acid, has been demonstrated by the xanthate reaction, though involvement of CoA would be difficult to prove without the use of cell-free systems, which are not readily obtainable from this organism. Our results were obtained by using mycelium grown on basal medium, so the enzyme system(s) involved are presumably 'constitutive'. The next stages presumably involve 'inducible' systems, as *o*-coumaric acid induces only its own conversion into 4-hydroxycoumarin, whereas melilotic acid induces conversion of both compounds.

The sequence of formation of radioactive products, whether ^{14}C- or ^{3}H-labelled, clearly supports the scheme suggested. The escape of substantial amounts of β-hydroxymelilotic acid and β-oxomelilotic acid into the medium suggests that the metabolites do not remain firmly bound to an enzyme surface throughout the sequence, as in the fatty acid β-oxidation system that our sequence superficially resembles. The results in Table 5 do not differentiate between intracellular and extracellular radioactive materials, but not more than 5% of the labelled material was in the mycelium in any one case, and the method of extraction would hydrolyse any thiol ester present. The reactive form may well be the ester, although no direct evidence for this is presented here.

It is difficult to explain the formation of *o*-coumaraldehyde. Perhaps the conditions of the reaction (semicarbazide followed by 2,4-dinitrophenylhydrazine at low pH) produce a distorted metabolism, in the presence of *o*-coumaric acid, with *o*-coumaraldehyde as an end product. Zenk (1965), however, suggests an analogous reaction for the conversion of benzoic acid (as the CoA derivative) into benzaldehyde in plants, though in this case he supplies no direct supporting evidence. Later work (Gross & Zenk, 1969) shows the reduction of various aromatic acids to the corresponding aldehydes and alcohols by cell-free preparations of *Neurospora crassa*.

Further work, involving the isolation of the enzyme(s) involved, is necessary to show the relation, if any, between the enzyme(s) involved in our reaction and those of the fatty acid β-oxidation.

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REFERENCES

Bellis, D. M., Spring, M. S. & Stoker, J. R. (1967). *Biochem. J.* **103**, 202.
Bocks, S. M. (1967). *Phytochemistry*, **6**, 127.
Bye, A., Ashton, W. M. & King, H. K. (1968). *Biochem. biophys. Res. Commun.* **32**, 94.
Dutt, S. (1924). *J. Indian chem. Soc.* **1**, 297.
Evans, E. A. (1966). *Tritium and its Compounds*, pp. 366–368. London: Butterworths Scientific Publications.
Fairbairn, N. J. (1953). *Chemy Ind.* p. 86.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Gross, G. G. & Zenk, M. H. (1969). *Eur. J. Biochem.* **8**, 413.

Levy, C. C. & Weinstein, G. D. (1964). *Biochemistry, Easton*, **3**, 1944.
Pauly, M. & Wascher, S. R. (1923). *Chem. Ber.* **56**, 606.
Roberg, M. (1948). *Arch. Mikrobiol.* **14**, 1.
Schmidt, O. T. & Treiber, R. (1933). *Chem. Ber.* **66**, 1769.
Seshadri, T. R. & Roa, S. (1954). *Chemy Ind.* p. 308.
Shieh, H. S. & Blackwood, A. C. (1967). *Can. J. Biochem. Physiol.* **45**, 2045.
Spring, M. S. & Stoker, J. R. (1968). *Can. J. Biochem.* **46**, 1247.
Spring, M. S. & Stoker, J. R. (1969). *Can. J. Biochem.* **47**, 301.
Stadtman, E. R. (1955*a*). In *Methods in Enzymology*, vol. 1, p. 596. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press.
Stadtman, E. R. (1955*b*). In *Methods in Enzymology*, vol. 3, p. 231. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press.
Stahl, E. (1965). *Thin Layer Chromatography*, pp. 162, 357 and 374–375. New York and London: Academic Press.
Stahman, M. A., Heubner, C. F. & Link, K. P. (1941). *J. biol. Chem.* **138**, 513.
Stanier, R. Y. (1951). *A. Rev. Microbiol.* **5**, 35.
Thompson, A. R. (1951). *Aust. J. Sci. Res.* B, **4**, 180.
Vogel, A. I. (1955). *A Textbook of Practical Organic Chemistry including Qualitative Organic Analysis*, 3rd ed., p. 704. London: Longmans, Green and Co.
Zenk, M. H. (1965). *Proc. FEBS 2nd Meet., Vienna*, p. 45.